Biomaterials 35 (2014) 4310–4318

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Modulation of matrix elasticity with PEG hydrogels to study melanoma drug responsiveness

Emi Y. Tokuda [a], Jennifer L. Leight [a,b], Kristi S. Anseth [a,b,*]

[a] Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO 80309, USA
[b] Howard Hughes Medical Institute and The BioFrontiers Institute, University of Colorado at Boulder, Boulder, CO 80309, USA

ARTICLE INFO

Article history:
Received 26 November 2013
Accepted 24 January 2014
Available online 22 February 2014

Keywords:
BRAF
Cell viability
ECM (extracellular matrix)
Elasticity
Hydrogel
Photopolymerization

ABSTRACT

Metastatic melanoma is highly resistant to drug treatment, and the underlying mechanisms of this resistance remain unclear. Increased tissue stiffness is correlated with tumor progression, but whether increased tissue stiffness contributes to treatment resistance in melanoma is not known. To investigate the effect of substrate stiffness on melanoma cell treatment responsiveness, PEG hydrogels were utilized as a cell culture system to precisely vary matrix elasticity and investigate melanoma cell responses to a commercially available pharmacological inhibitor (PLX4032). The tensile moduli were varied between 0.6 and 13.1 kPa (E) and the effects of PLX4032 on metabolic activity, apoptosis, and proliferation were evaluated on human cell lines derived from radial growth phase (WM35) and metastatic melanoma (A375). The A375 cells were found to be stiffness-independent; matrix elasticity did not alter cell morphology or apoptosis with PLX4032 treatment. The WM35 cells, however, were more dependent on substrate modulus, displaying increased apoptosis and smaller focal adhesions on compliant substrates. Culturing melanoma cells on PEG hydrogels revealed stage-dependent responses to PLX4032 that would have otherwise been masked if cultured strictly on TCPS. These findings demonstrate the utility of PEG hydrogels as a versatile *in vitro* culture platform with which to investigate the molecular mechanisms of melanoma biology and treatment responsiveness.

1. Introduction

Melanoma is an aggressive form of skin cancer that is difficult to treat in later stages by conventional chemotherapeutics. Early stage radial growth phase (RGP) melanoma is easily treated by surgical excision [1], while vertical growth phase (VGP) has a propensity to metastasize [2]. Metastatic melanoma, however, is notoriously drug resistant [3], characterized by a median survival time of 6–10 months [4]. One promising new drug, PLX4032 (Vemurafenib), was approved by the FDA in 2011 for metastatic melanoma treatment. PLX4032 is a small molecule inhibitor that has high specificity towards mutated BRAF proteins and has been shown to cause both cell cycle arrest and induce apoptosis in melanoma [5–7] and potent growth arrest in thyroid carcinoma cells [8,9]. More than 60% of all melanomas have been found to contain a BRAF mutation (V600E) that renders this protein constitutively active [10]. Clinical trials showed marked patient responses to PLX4032 [11,12] and follow up studies reported 16-month overall survival [13]. Yet while PLX4032 is among the most promising melanoma treatments, patients eventually relapse and the mechanisms and contributing factors of melanoma drug resistance remain elusive.

As one approach to aid in preclinical compound screening and better understanding of the molecular mechanisms that might contribute to melanoma drug resistance, improved *in vitro* culture systems are being explored. Traditional tissue culture-treated polystyrene (TCPS) is often the initial culture platform used for drug screening, but it is orders of magnitude stiffer than most soft tissues in the body and may lead to physiologically irrelevant cellular morphologies or responses [14–16]. Matrix elasticity has been shown to regulate cell function in a number of different cell types, such as mesenchymal stem cells [17] and smooth muscle cells [18], and clinically, tumors are often found to be stiffer than the surrounding or healthy tissues [19,20]. *In vitro*, increased substrate elasticity has been shown to induce malignant morphology in healthy mammary epithelial cells and lead to increased invasion of breast cancer cells [21,22]. The underlying substrate can also alter

* Corresponding author. Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO 80309, USA. Tel.: +1 303 492 3147; fax: +1 303 492 4341.
E-mail address: Kristi.Anseth@colorado.edu (K.S. Anseth).

http://dx.doi.org/10.1016/j.biomaterials.2014.01.063
0142-9612/

intracellular signaling [21,23], which ultimately may change the efficacy of drug treatments. In fact, Weigelt et al. showed that when breast cancer cells were cultured on TCPS or Matrigel, the reduction of proliferation to clinically available drugs was altered [24].

Many studies have shown the importance of matrix elasticity on breast cancer cells, but the same is not yet known for melanoma. Unlike epithelial-derived breast cancer cells, melanoma is derived from melanocytes which arise from the neural crest [25], and so it is difficult to assume melanocytes and epithelial cells will respond similarly to a microenvironmental change like substrate elasticity. We hypothesized that matrix elasticity is important for assessing melanoma responses to drug treatment and that softer materials may provide better insight into physiologically relevant cellular responses. To investigate melanoma's dependence on substrate modulus, we utilized peptide functionalized poly(ethylene glycol) (PEG) hydrogels as a highly tunable, hydrated, and chemically defined cell culture substrate that can be designed to recapitulate important aspects of the extracellular matrix (ECM) [26,27]. In particular, the thiol-ene "click" chemistry was exploited to form crosslinked networks via step-growth kinetics involving the reaction of an -ene functionalized multi-arm PEG with cysteine-containing peptides (-thiol) [28]. Cell–matrix interactions can be altered by the concentration of ECM molecule peptide mimics, such as the fibronectin-derived peptide RGDS [26]. Matrix remodeling can be controlled by inclusion of matrix metalloproteinase (MMP) degradable peptide sequences, allowing cell-mediated degradation [29]; alternatively, the hydrogel can also be rendered nondegradable by the inclusion of crosslinkers such as PEG-dithiols [30]. Finally, bulk biophysical properties, such as modulus or equilibrium water content, can be controlled by changing the network crosslinking density, which may be tuned by changing the concentration, molecular weight, or number of arms of the PEG [28,31]. This innate tunability of this biomaterial provides an attractive cell culture platform to answer fundamental questions about cellular responses to microenvironmental changes.

Here, we sought to answer whether matrix stiffness would alter melanoma cell morphology and responses to PLX4032 treatment using this synthetic ECM mimic. Formulations based on a 4-arm norbornene-functionalized PEG and bifunctional cysteine-containing MMP-degradable peptides were crosslinked using the thiol-ene photopolymerization approach. The matrix elasticity was varied from 0.6 to 13.1 kPa (E, Young's modulus) with the aim of spanning a range of mechanical properties reported for healthy and pathologic tissue, and the resulting gels were then seeded with either RGP or metastatic melanoma cells. Cell morphology and cell–matrix interactions were assessed via immunostaining and focal adhesion size then viability was challenged with PLX4032 treatment. To test cell responsiveness to this inhibitor as a function of the microenvironment, metabolic activity, apoptosis, and proliferation were quantified and correlated to substrate elasticity.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma–Aldrich unless otherwise noted. Cell culture reagents were purchased from Life Technologies unless otherwise noted. Antibodies used: monoclonal paxillin (Y113; Millipore) and goat anti-rabbit Alexa-Fluor 488. TRITC–phalloidin (Sigma–Aldrich) stocks were prepared at 0.06 mg/mL and DAPI stocks were 0.1 mg/mL. PLX4032 (ChemieTek) stocks were dissolved in DMSO (Sigma–Aldrich) at 100 mM.

2.2. Synthesis and characterization of macromers and peptides

4-arm PEG–norbornene (M_w: 20,000) (Fig. 1) was synthesized as previously described [28]. Briefly, norbornene acid was coupled to form norbornene anhydride in dichloromethane (DCM) via N,N′-diisopropylcarbodiimide (DIC) coupling. PEG hydroxyl (JenKem Technology, USA) was dissolved in DCM and reacted with the norbornene anhydride in the presence of pyridine and 4-(dimethylamino)pyridine (DMAP) overnight. The product was precipitated in cold diethyl ether 3 times, dried, and characterized by proton NMR for degree of functionalization. In all studies, PEG with >95% functionalization was used.

The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as previously described [32]. Briefly, 2,4,6-trimethylbenzoyl chloride was added to dimethyl phenylphosphonite (Acros Organics) and allowed to stir overnight. Lithium bromide and 2-butanone were then added to the reaction, heated to 50 °C for 10 min, and then cooled. The product was filtered, washed and filtered 3 times with 2-butanone, and allowed to dry. The product was verified by proton NMR.

All peptides were purchased from American Peptide Company, Inc. An MMP-degradable crosslinker (KCGPQG*IWGQCK) and pedant adhesion peptide RGD (CRGDS) were used.

2.3. Formation and characterization of PEG–norbornene hydrogels

Gels were formed between a glass slide that was dipped in Sigmacote and a thiolated coverslip to create flat hydrogels for cell seeding. Coverslips were passed through a flame to remove contaminates, and then reacted in 95% ethanol (pH ~5.5) with 0.55% (v/v) (3-mercaptopropyl)trimethoxysilane for 3 min. Coverslips were then rinsed with 95% ethanol and allowed to dry in an 80 °C oven for 15 min.

Macromer solutions were prepared with the concentrations of reagents in Table 1 with 1.7 mM LAP. In a sterile cell culture hood, 30 μL drops of macromer solution were placed on sterile Sigmacote-coated slides and then covered with a thiolated 18 mm circle coverslip (Fisher Scientific). The solutions were placed under a UV lamp centered around 365 nm light at ~5 mW/cm^2 for 3 min. The formed gels were then allowed to swell in PBS for at least 2 h, then sterilized with 5% IPA in PBS at room temperature for 1 h. Before cell seeding, the gels were rinsed twice with PBS. Unless otherwise noted, 3 gels were formed per condition for each of 3 independent experiments.

For characterization, 30 μL gels were formed in the cut-end of a 1 mL syringe. The same gel formulations as those used for cell culture were made (Table 1). To determine the equilibrium swollen gel mass, the gels were allowed to swell overnight in PBS then weighed the next day. The mass swelling ratio q (Table 2) was calculated from the equilibrium swollen mass and the calculated theoretical dry mass. The water content (% Water) was calculated using the mass swelling ratio $(1 - q^{-1})$ and converted to a percent. The shear modulus (G) was measured on a DHR-3 shear rheometer (TA Instruments) and converted to Young's modulus, where $E = 2G(1 + \nu)$ assuming a Poisson's ratio (ν) of 0.5 [33]. Strain and frequency sweeps were performed to ensure measurements were within the linear viscoelastic regime. Averages represent 3 independent experiments.

2.4. Cell culture

Both cell lines were a generous gift from Professor Natalie Ahn, Department of Chemistry and Biochemistry, University of Colorado. All cell lines were cultured in 10% fetal bovine serum (FBS) in RPMI 1640 without phenol red. For experiments, cells were seeded at 1×10^5 cells/cm^2 in 1% FBS in RPMI 1640 and subsequently cultured in 1% FBS for the remainder of any experiment. Cells were seeded on gels or TCPS and allowed to adhere overnight. DMSO control (0.01% v/v) or 1 μM PLX4032 diluted in media was then added to the corresponding samples and incubated for 48 h.

2.5. Metabolic activity

At the end of the 48-h drug incubation time, CellTiter-Glo (Promega) was added to samples as per the manufacturer's instructions. Samples were placed on an orbital shaker for 2 min, and then 100 μL of lysate was transferred to a white 96-well plate and the luminescence was measured on a BioTek H1 Synergy plate reader (BioTek). To measure basal metabolic activity, standard curves of ATP and DNA were generated. After applying CellTiter-Glo to samples, the cell lysate was then diluted to quantify DNA content by Quant-it PicoGreen (Life Technologies).

2.6. Apoptosis

After 48 h of PLX4032 treatment, the EnzChek Caspase-3 Assay Kit #2 (Life Technologies) was used to assess apoptosis. Briefly, floating cells were collected by centrifugation and attached cells were removed with TrypLE Select for 5–10 min at 37 °C and then agitated to remove as many cells possible. Cell pellets were then lysed with the provided lysis buffer and then the company protocol was followed. DNA content was measured via PicoGreen (Life Technologies), and caspase 3 activity was normalized to the DNA content in a given sample. For analysis, each PLX4032-treated sample was normalized to its corresponding DMSO control sample to show a fold change in caspase 3 activity with PLX4032 treatment.

2.7. Proliferation

During the last 16 h of incubation with or without the inhibitor, the cells were pulsed with EdU to detect cells entering S-phase. After a total of 48 h, samples were processed according to the protocol for the Click-iT EdU Alexa-Fluor 488 kit (Life Technologies). One exception was to invert the coverslips on 10 μL drops of the